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Theodore I. Malinin, M.D.
Principal Investigator

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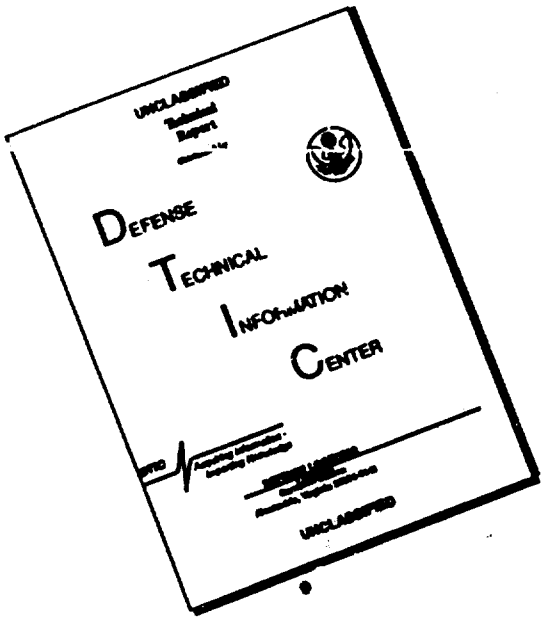

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Summary

The work performed on the contract dealt with prolonged and short-term perfusion of mammalian hearts of normothermic and hypothermic temperatures and with infusion of hypertonic cell free fluids into animals in hemorrhagic shock. In addition some aspects of freeze preservation of cardiac tissues and micro-vascular injury in perfusion were studied.

It was demonstrated in the course of the above investigations that excised primate hearts can be resuscitated by pulsatile perfusion with cell free fluids as long as 2 hours postmortem. The length of survival of hearts determined by independent rhythmical contractions depends on the temperature of perfusion. The longest survivals (average 9 days) were obtained with hearts perfused at 12-15° C. The shortest survival period (less than 12 hrs.) was observed with hearts perfused at 4-6° C.

Infusion of cell free solutions of various tonicities into animals in hemorrhagic shock demonstrated an extremely rapid adjustment of osmolality of the peripheral blood and the interstitial fluid. The recovery rate of animals infused with solutions in volumes equal to those of lost blood were of about 60% with hypertonic solutions and about 20% with lactated Ringer's solution. Addition of glutamyl polypeptide (plasma expanding colloid) resulted in the survival rate of about 90%.

Studies with cell dispersion in an effort to establish cultures of endothilium resulted in the development of a new technique for the dispersion of the animal tissues. This technique was based on concomitant use of disodium ethylene diamine and trypsin. The use of two dispersing agents enhanced cell yield as compared with standard cell dispersion methods. Moreover, significant reduction of time required for tissue dispersal, a low number of non-viable cells and a uniform monolayer formation on cultivation were obtained with this method.

Finding of metochromatic material in perfused hearts and kidneys of animals in shock led to the study of metochromatic staining. It was found that glycogen readily formed sodium bisulfite addition compounds following oxidation with periodic acid. Sodium bisulfite derivatives of oxidized glycogen developed intense metachromasia when stained with toluidine blue of the 1.0-5.0 pH range. It was concluded that the origin of the dehydration labile metachromasia resides with the external glucose units of glycogen depositions whereas the induction of dehydration resistant metachromasia may be attributed to the sodium bisulfite derivatives of the internally located glucosyl groups of glycogen.

Studies of the microvasculature of perfused isolated organs and of animals in hemorrhagic shock revealed patency of the microvasculature, and apparent alteration in the permeability of the capillary endothilium.

Studies with freezing of tissues were concentrated on perfusion with cryoprotective agents and to freezing of pieces of myocardium, whole hearts peripheral blood leukocytes and bone marrow. The latter study was performed in order to complete the studies initiated at the NNMC.

Perfusion of Whole Mammalian Hearts

Monkey hearts were subjected to pulsatile perfusion with hypertonic media (NCTC 135 with 2 x Earle's balanced salt solution). Perfusion was carried out in an all glass Lindbergh-Rockefeller Institute organ culture apparatus. Of one hundred and four simian hearts subjected to perfusion ninety-two responded with rhythmical atrial and ventricular contractions, while 12 did not. The hearts used in these experiments were removed from animal carcasses 2-3 hours after the death of the animal. Ten additional hearts were removed from animals few minutes after death. All of those responded to perfusion with strong atrial and ventricular contractions.

Adequacy of perfusion of hearts was assessed by direct observation of epicardial vessels, vital dye injection, vascular injection with silicone rubber compound, and histologic examination.

The ability of hearts to withstand perfusion with hypertonic solutions was tested. The hearts continued to pulsate while perfused through the coronaries with solutions containing as high concentration of electrolytes as 17g/l of NaCl, 1g/l KCl, 0.5g/l $MgSO_4$ and 5g/l of $CaCl_2$. Further increase in electrolyte concentration resulted in cardiac arrest as did an increase in concentration of KCl alone.

The ability of isolated hearts to maintain independent atrioventricular contractions for periods longer than 24 hours was related to the temperature of perfusion. Oxygenation of the media did not increase the period of survival although it produced transient increase in the force of contractions.

Repeated cardiac contractions resumed after consecutive coolings below the point of cardiac arrest although temperature at which contractions resumed was changed.

Experiments were performed to determine the most suitable temperatures for long-term perfusion of isolated hearts and to determine the length of survival of hearts in the apparatus.

Experiments were performed on more than 200 hearts from adult monkeys (Macaca mulatta and Cercophithecus aethiopus). The animals were sacrificed by an injection of an overdose of pentobarbital. The hearts were excised from the animals under sterile conditions, 1 to 2 hrs. postmortem. The hearts then were perfused in the Carrel-Lindberth apparatus at pressures of 100/80 mm Hg. The perfusing medium was NCTC 135 with 10% newborn calf serum and 2X concentrated Earle's balanced salt solution with 1X sodium bicarbonate.

Hearts were perfused in an identical manner with 37°C, 25 to 20°C, 15 to 12°C, and 4 to 6°C. Survival of the heart was assessed by direct observation of independent atrioventricular contractions, and by electrical activity recorded on EKG. Hearts cooled to temperatures below cardiac arrest were rewarmed every 12 hrs. for observation of contractions. The average survival time of hearts perfused at 37°C was about 24 hours; for those perfused at 25 to 20°C, about 48 hours. Hearts perfused at 12 to 15°C survived for an average of 10 days. Hearts perfused at 4 to 6°C survived less than 24 hours.

It is evident from these experiments that cardiac resuscitation was not directly related to postmortem period of between one to three hours. The animals used in these experiments were heparinized prior to sacrifice and the carcasses were refrigerated soon thereafter. Since the animals were small, usually 6-8 pounds, they cooled rapidly. Similar results with post-mortem intervals of up to six hours and the resuscitation of adult human cadaver hearts were reported by Andreev (1953).

Gross observations of hearts perfused with so called "isotonic" solutions and the distention of interstital spaces, observed on microscopic examination, demonstrated accumulation of excess extravascular interstitial fluid in these hearts. The rapidity with which vital dyes injected into coronary arteries stained the entire myocardium suggested increased vascular wall permeability. Perfusion with salt solutions containing twice the amount of inorganic salts found in serum partially decreased the edema as did the reduction of the temperature.

Direct microscopic observation of subendocardial blood vessels revealed the absence of erythrocytes in these vessels, but hemorrhagic foci in the myocardium persisted throughout the entire course of perfusion. Microscopic

observation likewise revealed the absence of red blood cells in the capillaries or the small blood vessels of the majority of perfused hearts, as well as no free hemoglobin demonstrable by benzidine or the Dunn-Thompson methods. This observation suggested adequate perfusion of the microvascular bed and the wash out rather than destruction of erythrocytes. Some hearts, however, contained areas with red blood cells in the capillaries and the interstitial spaces. In such instances the red blood cells were accompanied by lymphocytes and these persisted for as long as perfusion was carried out. The erythrocytes were not numerous enough to be conspicuous, and were primarily found in the interstitial spaces following break up of the myocardial structures and the capillaries. This finding suggested trapping of RBC's and lymphocytes in nonperfused capillaries.

Reduction of temperature from 37° to 22°-25°C almost doubled the survival time of perfused hearts. Lowering of perfusion temperature below the point of cardiac arrest to 12°-15°C prolonged the survival time to about ten days. Further reduction of temperature, however, not only failed to further increase the survival time, but actually reduced it. Notwithstanding the fact that the hearts were not contracting at 4°-6°C, they did not even survive as long as continuously contracting hearts perfused at 37°C.

Histological observations on the myocardium of hearts perfused in this series of experiments showed that pathological changes could be correlated with heart contractility. These changes were quite apparent in hearts fixed after contractions had ceased or were markedly diminished. The appearance of earlier changes depended on temperature of perfusion. Changes consisting of fragmentation of the myofibrils with appearance of the pyroninophilic globules, separation of myocardial fibers, clumping of protein, and bulging

of the sarcoplasm with the rupture of the myocardial fibers were evident as early as six hours after initiation of contractions in hearts perfused at 22°-25°C. In hearts perfused at 12°-15°C, early changes were noted only after 24-48 hours of perfusion. The fragmentation of the myocardial fibers was a rather common form of injury in the perfused hearts. It was apparently preceded by condensation of the protein in the sarcolemma, weakening of the membrane, and protrusion of the sarcoplasmic mass into the interstitial spaces.

Ultrastructural changes reminiscent of ischemic, presumably reversible changes of short duration, were observed in control hearts. Similar changes were observed by other investigators in dog cadaver hearts incubated at 37°C. The fibrils were relaxed and the interfibrillar spaces increased some elements of the sarcoplasmic reticulum were indistinct. The borders of the mitochondria were clearly defined, but many mitochondria were surrounded by clear spaces. The cristae in a few mitochondria were dilated.

Specimens of myocardium obtained from hearts perfused for 6 hours of 22-25°C showed distention of mitochondrial cristae and no visible matrix. The mitochondria were clumped in the perinuclear region. The nuclei contained dense heterochromatin at the nuclear membrane and euchromatin within the nuclei. The nucleoli appeared normal. In some portions of the myocardial fibers the myofibrils were grossly distorted and little could be described of the filaments and the normal banding pattern. The mitochondria from the perinuclear region of hearts perfused for 24 hours at room temperature showed distention of cristae and the loss of matrix density. The ultrastructural architecture of hearts with marked diminution or cessation of contractile activity was grossly distorted. Most mitochondria were swollen and vacuolated and showed a loss of the matrix substance. The myofibrillar filaments or the banding pattern were difficult to recognize.

Cryoprotective Agents

The action of propylene glycol, dimethyl sulfoxide, and glycerine on hearts perfused *in vitro* was tested.

Monkey and rabbit hearts were placed in the apparatus and perfused with NCTC 135 and 10% newborn calf serum until independent rhythmical cardiac contractions were established. All perfusions were carried out at room temperature (22° to 30°C). Protective agents were then added to the perfusate in 5% by volume increments. The addition of cryoprotective agents was continued at 10-minute intervals until cessation of cardiac contractions. When this occurred the cryoprotective agent was washed out by complete fluid change, perfusion continued, and the heart observed for resumption of mechanical activity.

The results of these experiments demonstrated that cardiac activity can be maintained in hearts perfused with dimethyl sulfoxide in concentrations up to 20% by volume. Perfusion with higher concentrations of dimethyl sulfoxide resulted in complete cessation of cardiac contractions. Autoradiographic studies with C^{14} labelled dimethyl sulfoxide indicated that most of the labelled compound remained in the interstitial spaces and was not in the cells.

The studies with propylene glycol indicated that it is toxic to the hearts *in vitro* in concentration of 25% by volume and above.

Experiments with glycerine were not satisfactory. It failed to dissolve satisfactorily in the perfusate, and continually settled down on the bottom of the vessel. It was therefore deemed unsuitable for use with the perfusion apparatus employed in this study.

Infusion of Cell Free Fluids into Animals in Hemorrhagic Shock

The study was performed on anesthetized white rabbits weighing between 1.7 and 3.2 kg.

Hemorrhagic shock was produced by initial rapid venous bleeding through the femoral veins of the animals until a blood pressure of 30 mm Hg was reached. Slow bleeding was continued for the next 15-20 minutes. The total amount of blood removed from each animal was between 30-33ml/Kg or about 60% of blood volume. During this final bleeding period blood pressure was usually between 5-10 mm Hg. Blood pressure was monitored directly by placing a cannula into the aorta via the femoral artery and connecting it to a mercury manometer. Usually at the end of the bleeding period the animals had stopped breathing or were demonstrating acute respiratory distress. The pupils were widely dilated. After the final fall of blood pressure most animals developed involuntary athetoid limb movements and defecatory motions in addition to the above mentioned clinical signs. Reinfusion of the selected test solution was started when these signs appeared and always after 20 minutes of arterial pressure below 30 mm Hg. Reinfusion time averaged 10 to 15 minutes. The animals were reinfused with (1) 10% low molecular weight Dextran in saline (328 milliosmols) (2) Hank's balanced salt solution (HBSS) diluted with equal volume of physiological saline (285 milliosmols) (3) 1.25% glutamylpolypeptide dissolved in solution as in (2) /300 milliosmols), (4) lactated Ringer's solution (257 milliosmols).and (5) 1/2 x (156 milliosmols) 1X (265 milliosmols) 2X (524 milliosmols) 3X (995 milliosmols) and 4X (1101 milliosmols) Hank's balanced salt solution.

Seventy percent of animals reinfused with LMW dextran and HBSS/saline died, and thirty percent survived. Eighty percent of animals infused with

lactated Ringer's solution died. There were no survivors in the groups of animals infused with 1/2X and 1X HBSS. Survivors in the 2X and 3 were in the 30 percent range. Sixty percent of animals infused with 4X HBSS survived. In the group of animals injected with glutamylpolypeptide there was a 90% survival rate.

The rabbit was chosen as an experimental animal in these studies because of its well recognized poor tolerance to shock. This animal permitted standardization and relative reproducability of findings with regard to the degree of shock within a limited time span of a few hours.

The replacement of blood by the employed fluids was by the venous route. Therefore, results obtained in this study cannot be compared with results obtained by more elaborate resuscitative efforts such as intra-arterial infusion, counter pulsation or the like.

Data obtained so far suggests that the polyglutamic acid in the concentration used is considerably more effective in sustaining life in experimental animals following hemorrhage than other solutions used in this study.

Administration of hypertonic salt solutions was followed by readjustment of osmolarity of the blood. By the time infusion was completed regardless of whether osmolarity of the solution was 260 or 1100 milliosmols, blood osmolarity remained in the 340 and 360 range. This suggested dilution of intravascular fluid with fluid from the extravascular compartment, which would of course imply selective permeability of capillary endothelium. However, after 30 min. to 60 min. the osmolarity began to rise again. This suggests final fluid readjustment.

Dispersion of Tissues for Preparation of Cell Cultures

The cell yields obtained with the trysin-disodium EDTA cell dispersion method were far in excess of the cell yields reported by other workers.

The increase of cell yields obtained with the trypsin-disodium EDTA method was not due to systematic errors in the count, as reflected by the fact that diluting the cell suspension to as low as 7×10^4 cells per ml of confluent cell monolayers were still obtained in 7 days. Apart from the increase in the cell yield, fewer dead cells and cell clumps were noted. These data compare favorably with the results obtained with the method of Youngner. Decreases in the percentage of dead cells could be most likely attributed to: (i) reduction in the duration of the dispersion procedure, or (ii) the elimination of the cell centrifugation in the conical tube for determination of the total cell volume.

Cell cultures routinely prepared with the trypsin-disodium EDTA technique now have been in use for over 2 years by different laboratories. No detectable differences were noted as compared with cells prepared with other methods. The cell monolayers prepared with trypsin-disodium EDTA dispersion have less debris and a more uniform cell layer, consequently making it easier to observe cytopathogenic effects.

It was observed that young rabbits gave a much higher cell yield than the older animals.

No attempt is made to explain the mechanism of increased cell yield obtained with the trypsin-disodium EDTA method. In spite of the fact that disodium EDTA has been used for dispersing monolayer cultures of established cell lines, we were not able to disperse renal tissues with disodium EDTA alone, and a few cells obtained were dead, most likely due to the extended time required to effect even partial dispersion.

Tissue Metachromasia

In fixed tissues and cell cultures glycogen readily formed sodium bisulfite addition compounds following oxidation with 0.5% aqueous periodic acid. Sodium bisulfite derivatives of oxidized glycogen developed intense metachromasia if stained with 0.5% toluidine blue at the 1.0-5.0 pH range. Induced metachromasia of tissue glycogen was dehydration-labile if the oxidation with periodic acid was carried out for less than 15 min. Oxidation in excess of 15 min. resulted in formation of dehydration-resistant metachromasia. The induction of the two types of metachromasia was determined primarily by the duration of oxidation and only secondarily by the duration of addition reaction and sodium bisulfite concentration. The origin of the dehydration-labile metachromasia probably resides with the external glucose units of glycogen depositions, whereas the induction of dehydration-resistant metachromasia may be attributed to the sodium bisulfite derivatives of the internally located glucosyl groups of glycogen.

Freezing of Hamster Hearts

Relationship between the length of time allowed for ice to form at a given freezing temperature and the ability of frozen tissues to recover after thawing, was studied. Excised hamster hearts were frozen at -1.5, -2 or -3°C in the absence of the cryoprotective agents for times corresponding to 1/4, 1/2, 3/4 or full length of the plateu of the freezing curve. As judged by the strength of contraction and the survival time after thawing, freezing at -3°C for 11 min. (full plateu) did not permit recovery. Freezing at -1.5°C for 6.25 min. (one-fourth plateu) produced a close to normal recovery.

It was concluded that there is a lower limit of temperature at which the congelation of all freezable water results in the relatively short time with a resultant production of lethal injury.

Long Term Storage of Bone Marrow

Dogs subjected to lethal whole body irradiation were transplanted with autologous bone marrow which had been stored in liquid nitrogen for up to three years. The animals were followed for up to five years after irradiation and transplantation.

The animals used in this study were male Beagles between one and two years of age. A total of 28 animals were subjected to irradiation. Of these, 15 underwent bone marrow transplants and 13 served as controls. Prior to initiation of experiments the animals were immunized against hepatitis, leptospira and distemper. Animals were dewormed, and routine blood analyses were performed. Bone marrow was collected from anesthetized animals by multiple needle aspiration of ribs and long bones. Approximately 20-30 ml. of bone marrow aspirate was collected from each animal. These were mixed with an equal volume of heparinized tissue culture medium 199. The suspension was then centrifuged and a 30% solution of DMSO prepared with supernatant fluid. The packed cells were resuspended in an equal volume of this solution, placed in special flat glass containers, cooled at rate of 1°C to - 30°C and then at 10°C per minute to -100°C. Frozen marrow was stored in the vapor phase of liquid nitrogen for up to three years. The bone marrow obtained from each animal contained between 2.5 to 8.2 x 10^9 nucleated cells. One sample of marrow was stored 12 days in liquid nitrogen, five were stored for one year, and nine were stored for three years. Smears were prepared from each sample of bone marrow following thawing. At the end of the storage period of bone marrow, the animal from which it was obtained received 700r x-ray total body irradiation via two lateral exposures from a Vande Graaf Generator Therapy Unit. Thawed bone marrow was injected intravenously into the original donor 24 hours following radiation exposure. Post-irradiation care consisted only

of administration of antibiotics. Control animals were treated in an identical fashion, save for administration of bone marrow.

The laboratory studies performed on the animals during post-irradiation period included CBC, platelet and reticulocyte counts. After return of the WBC to pre-irradiation levels the animals were transferred to outdoor runs and maintained there until time of sacrifice. Hematologic studies and routine clinical chemistries were performed before the dogs were killed by an overdose of Nembutal. A complete autopsy was performed on sacrificed animals.

All control animals died between 10 and 21 days post-irradiation. Of 15 irradiated animals reinfused with frozen stored bone marrow, 12 survived and three died. All three animals in the group which died showed histologic evidence of bone marrow regeneration. The animals which were sacrificed at 2, 3½ and six months after irradiation and bone marrow transplantation showed pathological changes in the testes and lymphoid tissue. Animals sacrificed 2 and 5 years after irradiation exposure contained changes only in some seminiferous tubules in the testes. Lymphoid tissue had assumed normal appearance. Hematologic and biochemical examination of peripheral blood from 2 and 5 year survivals was within normal limits, and the animals appeared healthy. The chromosomes from a 5 year survival appeared normal, and did not show breakage. Long-term surviving males, when bred with non-irradiated females, produced normal offspring. Malignancies were not found in any animals.

Cytological examination of bone marrow cells for 1 and 3 years demonstrated atypical changes in 3 to 25% of cells. However, the distribution of altered cells was extremely irregular and could not be correlated with the length of storage. The remaining cells maintained normal architecture.

In summary, the reported experiments demonstrate the practicality of long-term storage of bone marrow at liquid nitrogen temperatures. However, the failure to protect 3 out of 15 animals from post-irradiation hematopoetic death under ideal conditions of autologous transplantation calls for some caution and re-evaluation of existing freezing and storage techniques. Bone marrow is a complex tissue composed of several different elements with different functions. Therefore, when attempts are made to refine and improve freeze-preservation techniques, this must be taken into consideration.

Complete recovery and the lack of serious post-irradiation complication in animals followed for five years are noted.

Infusion of Frozen Stored Autologous Bone Marrow into Lethally Irradiated (700r) Dogs

mal No.	Cell Dose	Time in Storage	Survival Post-Irradiation	Cause of Death
(12 Kg)	4×10^9	3 years	10 days	Hypoplastic marrow,[1] Hemorrhagic Pneumonia
(12 Kg)	5.4×10^9	1 year	17 days	Hypoplastic marrow,[1] Septicemia
(14 Kg)	4×10^9	1 year	23 days	Hypoplastic marrow,[1] Septicemia
(13 Kg)	8.2×10^9	3 years	2 months	Sacrificed
(14 Kg)	7.0×10^9	3 years	2 months	Sacrificed
(8 Kg)	7.0×10^9	3 years	3½ months	Sacrificed
(9.8 Kg)	6.8×10^9	3 years	3½ months	Sacrificed
(12 Kg)	6.4×10^9	3 years	6 months	Sacrificed
(10 Kg)	3.6×10^9	3 years	6 months	Sacrificed
(9 Kg)		3 years	2 years	Accidental Poisoning
(8.8 Kg)	3.0×10^9	3 years	2 years	Sacrificed
(15 Kg)	5.5×10^9	1 year	5 years	Sacrificed
(12 Kg)	6.2×10^9	1 year	5 years	Sacrificed
(8 Kg)	2.5×10^9	12 days	5 years	Sacrificed
(13 Kg)	3.5×10^9	1 year	5 years	Sacrificed

ows evidence of bone marrow regeneration.

Publications Resulting From Work on Subject Contract

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